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DNA Fingerprinting of *Vibrio cholerae* Strains with a Novel Insertion Sequence Element: a Tool To Identify Epidemic Strains

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A novel *Vibrio cholerae* insertion sequence element, designated IS*1004*, was characterized and used for DNA fingerprinting of *Vibrio* spp. IS*1004* comprises 628 bp and contains an open reading frame whose product shows a large degree of sequence identity with the IS*200*-encoded transposase. IS*1004* was present in one to eight copies in most of the *V. cholerae* strains analyzed. The IS*1004*-generated fingerprints of epidemic *V. cholerae* strains with serotype O1 were closely related, although it was possible to distinguish between the two biotypes, classical and El Tor. Non-O1 serotype strains generally showed heterogeneous patterns unrelated to those of the epidemic O1 strains. Several strains were observed with identical or related fingerprint patterns but expressed different serotypes. Conversely, strains with different fingerprint patterns but identical serotypes were also found. These observations indicate that the gene clusters coding for distinct O antigens may be transferred horizontally between *V. cholerae* strains. Two examples of non-O1 strains with a fingerprint resembling that of epidemic O1 strains were found; they were the O139 Bengal strain and an O37 strain. The O139 Bengal strain is closely related to the El Tor biotype. The O37 strain was responsible for a large cholera outbreak in Sudan in 1968 and was classified as a noncholera vibrio. Our study, however, shows that the O37 Sudan strain is genetically closely related to classical O1 strains. Similar to O139 Bengal, O37 Sudan lacked most of the O1 antigen cluster but did contain flanking genes. Thus, O37 Sudan represents a second example of an epidemic *V. cholerae* strain carrying non-O1 antigens. This study underlines the importance of genotypic methods for the differentiation of *V. cholerae* strains and for the recognition of strains with epidemic potential.

To date, more than 140 *Vibrio cholerae* serotypes have been identified, but the three cholera pandemics recorded since 1881 were exclusively caused by strains of serotype O1 (4, 34). The O1 serotype can be further subdivided into the classical and El Tor biotypes on the basis of phenotypic differences. The ongoing pandemic, which started in 1961, is caused by the El Tor biotype, while strains isolated from the two previous pandemics (1881 to 1896 and 1899 to 1923) were of the classical biotype (4). *V. cholerae* serotypes other than O1 (usually called non-O1) are only sporadically pathogenic for humans (25, 33). Some non-O1 strains, however, produce cholera toxin, and these strains can cause severe, cholera-like symptoms (20, 25, 26, 31). Until recently, though, non-O1 strains have been associated only with small outbreaks or isolated cases of intestinal or extraintestinal infections and were regarded to lack epidemic potential (19, 25, 33). Therefore, it was totally unexpected that a large cholera outbreak which started in 1992 in India and Bangladesh was caused by a non-O1 *V. cholerae* strain with serotype O139, with the synonym Bengal (reviewed in reference 1). The disease had all the characteristics of cholera caused by the O1 serotype, and there is now ample evidence that the O139 Bengal strain has evolved from a *V. cholerae* O1 El Tor strain by an exchange of genes coding for synthesis of cell surface polysaccharides (6, 12).

The emergence of the O139 strain illustrates that serotyping is of limited value for predicting the epidemiological potential of a strain and underlines the importance of genotypic methods. Several genotypic approaches to differentiate between *V. cholerae* strains, such as multilocus enzyme electrophoresis, ribotyping, and pulsed-field gel electrophoresis (9, 13, 21, 42), have been used. Of these techniques, pulsed-field gel electrophoresis appears to have the best discriminatory potential. However, this method is quite laborious and the large number of bands makes the interpretation of results and comparison between different laboratories or even different gels difficult. Another genotypic method which has been applied successfully for strain differentiation is DNA fingerprinting using insertion sequence (IS) elements. The restriction fragment length polymorphism associated with the presence of multiple IS elements has been applied successfully for the differentiation of strains of a variety of bacterial species, but not for *V. cholerae*. In this report, we describe a novel *V. cholerae* IS element and its use to differentiate between *V. cholerae* strains by DNA fingerprinting. Using this approach, we identified a second example of a non-O1 serotype strain with the genetic background of an epidemic strain.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *V. cholerae* strains and other *Vibrio* isolates used in this study are described in Table 1. *Escherichia coli* DH5α (Gibco BRL, Gaithersburg, Md.) was used for the cloning of DNA fragments. All plasmids used in this study are listed in Table 2. The sequences of primers used in this study are listed in Table 3.

DNA techniques. Chromosomal DNA extraction, digestion, and cloning were performed as described by Ausubel et al. (3). Restriction endonucleases were from Boehringer Mannheim. PCR was performed as described by Bik et al. (6). Southern blotting was done as described by van Soolingen et al. (41). Probes for Southern blot hybridization were isolated by using Qiaex or Qiaquick DNA purification kits (Qiagen Inc., Chatsworth, Calif.) and labeled by using the enhanced chemiluminescence gene detection system (Amersham International PLC, Amersham, United Kingdom).

Cloning and sequencing of IS*1004*. We fortuitously identified a repeated DNA

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TABLE 1. *Vibrio* strains used in this study

Strain	Biotype	Serotype	Relevant characteristic(s)	Cholera toxin production[a]	Source and/or reference[b]	Fingerprint type
V. cholerae						
CVD101	Classical	O1 Ogawa	*ctxA* mutant of 395, vaccine strain	−	18	C1
395	Classical	O1 Ogawa	Patient isolate, India	+	10	C1
569B	Classical	O1 Inaba	Patient isolate, 1948, India	+	10	C2
NCTC 8039	Classical	O1 Inaba	Patient isolate, 1955	+	RIVM	C1
C21	Classical	O1 Ogawa	Patient isolate, 1963, Pakistan	+	17	C1
Cairo 48	Classical	O1 Inaba	Patient isolate, 1949, Egypt	+	RIVM	C1
C5	El Tor	O1 Ogawa	Patient isolate, 1957, Indonesia	+	17	E1
C30	El Tor	O1 Ogawa	Patient isolate, 1981, Tunesia	+	RIVM	E1
N16961	El Tor	O1 Inaba	Patient isolate, <1982, Bangladesh	+	10	E2
35473	El Tor	O1 Inaba	Patient isolate, 1992, Venezuela	+	INHRR	E3
35481	El Tor	O1 Inaba	Patient isolate, 1992, Venezuela	+	INHRR	E3
35518	El Tor	O1 Inaba	Patient isolate, 1992, Venezuela	+	INHRR	E3
35656	El Tor	O1 Inaba	Patient isolate, 1992, Venezuela	+	INHRR	E3
35730	El Tor	O1 Inaba	Sewage isolate, 1992, Venezuela	+	INHRR	E3
35757	El Tor	O1 Inaba	Sewage isolate, 1992, Venezuela	+	INHRR	E3
35759	El Tor	O1 Inaba	Sewage isolate, 1992, Venezuela	+	INHRR	E3
37188	El Tor	O1 Inaba	Patient isolate, 1992, Venezuela	+	INHRR	E3
38068	El Tor	O1 Ogawa	Patient isolate, 1992, Venezuela	+	INHRR	E3
38358	El Tor	O1 Ogawa	Patient isolate, 1992, Venezuela	+	INHRR	E3
38359	El Tor	O1 Ogawa	Patient isolate, 1992, Venezuela	+	INHRR	E3
38578	El Tor	O1 Ogawa	Patient isolate, 1992, Venezuela	+	INHRR	E3
9400377	El Tor	O1 Ogawa	Patient isolate, 1994, Rwanda	+	RIVM	E1
9400378	El Tor	O1 Ogawa	Patient isolate, 1994, Rwanda	+	RIVM	E1
V83	El Tor	O1	Patient isolate, 1994, Indonesia	+	AMC	E1
CO440	El Tor	O1	Patient isolate, 1994, India	+	IMCJ	E1
CO443	El Tor	O1	Patient isolate, 1994, India	+	IMCJ	E1
CO447	El Tor	O1	Patient isolate, 1994, India	+	IMCJ	E4
CO457	El Tor	O1	Patient isolate, 1994, India	+	IMCJ	E1
CO458	El Tor	O1	Patient isolate, 1994, India	+	IMCJ	E4
CO460	El Tor	O1	Patient isolate, 1994, India	+	IMCJ	E1
CO462	El Tor	O1	Patient isolate, 1994, India	+	IMCJ	E1
CO471	El Tor	O1	Patient isolate, 1994, India	+	IMCJ	E1
5188	El Tor	O1 Ogawa	Patient isolate, 1995, Zaire	+	AMC	E1
5189	El Tor	O1 Ogawa	Patient isolate, 1995, Zaire	+	AMC	E1
5190	El Tor	O1 Ogawa	Patient isolate, 1995, Zaire	+	AMC	E1
MO45/ATCC 51394		O139	Patient isolate, 1993, India	+	KU (27)	E5
NT552		O139	Patient isolate, 1993, India	+	NICED	E5
NT554		O139	Patient isolate, 1993, India	+	NICED	E5
NCTC 4711		O2	O reference strain	−	NIHT (34)	N1
NCTC 4715		O3	O reference strain	−	NIHT (34)	N2
NCTC 4716		O4	O reference strain	−	NIHT (34)	N3
B4202-64		O5	O reference strain	−	NIHT (34)	N4
7007-62		O6	O reference strain	−	NIHT (34)	N5
8394-62		O7	O reference strain	−	NIHT (34)	N6
10317-62		O8	O reference strain	−	NIHT (34)	N7
112-68		O9	O reference strain	−	NIHT (34)	N8
218-68		O10	O reference strain	−	NIHT (34)	N9
10843-62		O11	O reference strain	−	NIHT (34)	N10
SG2		O60	Patient isolate, 1992–1993, India	−	NICED	N11
SG3		O32	Patient isolate, 1992–1993, India	−	NICED	N12
SG4		O2	Patient isolate, 1992–1993, India	−	NICED	N13
SG6		O45	Patient isolate, 1992–1993, India	−	NICED	N14
SG7		O56	Patient isolate, 1992–1993, India	−	NICED	—[c]
SG8		O37	Patient isolate, 1992–1993, India	−	NICED	—
SG10		O69	Patient isolate, 1992–1993, India	−	NICED	N15
SG13		O24	Patient isolate, 1992–1993, India	−	NICED	N16
SG14		O54	Patient isolate, 1992–1993, India	−	NICED	N17
2107-78		Rough	Patient isolate, 1978, Bangladesh	+	CDC	C1
3176-78		O141	Water isolate, 1978, Georgia	+	CDC	N18
609-84		O141	Patient isolate, 1984, New York	+	CDC	N19
2454-85		O141	Patient isolate, 1985, Tennessee	+	CDC	N19
2466-85		O141	Patient isolate, 1985, North Carolina	+	CDC	N19
2533-86		O141	Patient isolate, 1986, California	+	CDC	N19
2527-87		O141	Patient isolate, 1987, Maryland	+	CDC	N19
E8498		O141	Environmental isolate, 1978, Louisiana	+	CDC	N18
S7		O37	Patient isolate, 1968, Sudan	+	CDC (28, 43)	C3

Continued on following page

TABLE 1—*Continued*

Strain	Biotype	Serotype	Relevant characteristic(s)	Cholera toxin production[a]	Source and/or reference[b]	Fingerprint type
G12R		O37	Patient isolate, 1968, Sudan	+	CDC (43, 44)	C3
FY2G		O8	Patient isolate, Thailand?	+	CDC	N20
9300028		Non-O1	Patient isolate, 1993, The Netherlands	−	RIVM	N21
9300031		Non-O1	Patient isolate, 1993, The Netherlands	−	RIVM	N22
9300169		Non-O1	Patient isolate, 1993, The Netherlands	−	RIVM	N23
9300268		Non-O1	Patient isolate, 1993, The Netherlands	−	RIVM	—
9300575		Non-O1	Shrimp isolate, 1993, The Netherlands	−	RIVM	N24
9300576		Non-O1	Shrimp isolate, 1993, The Netherlands	−	RIVM	N24
36328		Non-O1	Sewage isolate, 1992, Venezuela	ND[d]	INHRR	N25
36309		Non-O1	Sewage isolate, 1992, Venezuela	ND	INHRR	N26
40106		Non-O1	Patient isolate, 1993, Venezuela	ND	INHRR	N27
S165		Non-O1/non-O139	Patient isolate, 1995, The Netherlands	−	RIVM	—
Other *Vibrio* species						
ATCC 33653		ND	*V. mimicus* reference strain	ND	NIHT	N28
ATCC 33809		ND	*V. fluvialis* reference strain	ND	NIHT	—
ATCC 33564		ND	*V. hollisae* reference strain	ND	NIHT	—
4750		O2K3	*V. parahaemolyticus* reference strain	ND	NIHT	—
VM-1		UT[e]	*V. mimicus*	−	NICED	N29
VM-2		O41	*V. mimicus*	−	NICED	N21
VM-4053		O126	*V. mimicus*	+	NICED	N30
VM-4208		O32	*V. mimicus*	−	NICED	N31
VM-4197		O34	*V. mimicus*	−	NICED	—

[a] +, production; −, lack of production.
[b] RIVM, National Institute of Public Health and the Environment, Bilthoven, The Netherlands; INHRR, M. C. de Chacón and S. de Waard, Instituto Nacional de Higiene "Rafael Rangel," Caracas, Venezuela; AMC, AMC Hospital, Amsterdam, The Netherlands; IMCJ, Y. Takeda and G. B. Nair, International Medical Center of Japan, Tokyo, Japan; KU, Y. Takeda, Department of Microbiology, Faculty of Microbiology, Kyoto University, Kyoto, Japan; NICED, G. B. Nair and M. K. Bhattacharya, National Institute of Cholera and Enteric Disease, Calcutta, India; NIHT, T. Shimada and Y. Takeda, National Institute of Health, Tokyo, Japan; CDC, J. C. Feeley, Centers for Disease Control and Prevention, Atlanta, Ga.
[c] —, no hybridization with IS*1004* probe.
[d] ND, not determined.
[e] UT, untypeable.

element when characterizing a Tn*phoA* mutant of *V. cholerae* CVD101 which was auxotrophic for δ-aminolevulinic acid (32). The region harboring the transposon in this mutant was cloned from CVD101 and used for Southern blotting. Unexpectedly, this region, contained as a 2.0-kb *Cla*I fragment in pEB1056, hybridized to multiple chromosomal bands, indicating that it harbored a repetitive DNA element. The repeat could be mapped to a 300-bp *Ava*II-*Cla*I fragment derived from the insert of pEB1056. This 300-bp fragment was subcloned, and the resulting plasmid was designated pEB1062. To clone different copies of the repetitive element, CVD101 chromosomal DNA was digested with *Hpa*II. Fragments of sizes corresponding to fragments hybridizing to pEB1056 were isolated from an agarose gel by using a Qiaex purification kit (Qiagen), blunted with Klenow polymerase, and ligated into the *Sma*I site of pEMBL18. Ligation mixtures were transformed into *E. coli* DH5α and plated onto NZ agar plates with ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and isopropyl-β-D-thiogalactopyranoside (IPTG) (3). White colonies were screened on Hybond-N filters with the enhanced chemiluminescence-labeled insert of pEB1056 (Amersham International PLC). Twenty hybridizing colonies were selected, and plasmid DNA of these colonies was isolated. After digestion and electrophoresis of plasmid DNA, fragments were transferred to Hybond-N^+ membranes (Amersham International PLC) and probed with the insert of pEB1062. Five plasmids were selected for further analysis. Plasmids pEB1107, -1109, -1110, -1111, and -1113 contained *Hpa*II inserts of 1,900, 1,500, 2,500, 1,500, and 2,200 bp, respectively. Plasmid pEB1109 and pEB1111 contained inserts of the same length but comprised different *Hpa*II fragments, as was concluded by restriction analysis (data not shown). Therefore, the 1,500-bp *Hpa*II fragment hybridizing to the insert of pEB1062 (see Fig. 1) is a double band. The insert of pEB1110 overlapped with that of pEB1056. Two internal *Cla*I sites in the pEB1110 insert were used to make subclones for sequencing.

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristic(s)	Reference
pEMBL18	Cloning vehicle; ampicillin resistance	14
pEB1056	pEMBL18 with 2,040-bp *Cla*I chromosomal fragment from CVD101	This study
pEB1062	pEMBL18 with 300-bp *Ava*II-*Cla*I fragment of pEB1056 insert	This study
pEB1107	pEMBL18 with 1,900-bp *Hpa*II chromosomal fragment from CVD101 containing IS*1004* copy C	This study
pEB1109	pEMBL18 with 1,500-bp *Hpa*II chromosomal fragment from CVD101 containing IS*1004* copy B	This study
pEB1110	pEMBL18 with 2,500-bp *Hpa*II chromosomal fragment from CVD101 containing IS*1004* copy E	This study
pEB1111	pEMBL18 with 1,500-bp *Hpa*II chromosomal fragment from CVD101 containing IS*1004* copy A	This study
pEB1113	pEMBL18 with 2,200-bp *Hpa*II chromosomal fragment from CVD101 containing IS*1004* copy D	This study

TABLE 3. Primers used in this study[a]

Primer	Sequence (5′ to 3′)	Positions of primer
9	ATAAAAATCCGCCTTCTTAG	1–20 of IS*1004*
10	ATTGTCATCCCTAAACCACC	625–605 of IS*1004*
72	ATCAACGTCGAAATCGATAC	300–320 of IS*1004*
73	CATGTCCACTTAGTTGCGAT	340–320 of IS*1004*
31	GGATAGGGCCATCAAAATAT	55–74 of X59554
78	GAACTTCAAACGTGATTTCG	552–533 of X59554
27	TTTGAAGGATGGCGTTTTA	4273–4291 of X59554
28	TTATTGCTTGAGAATCGCC	7072–7054 of X59554
18	CCAGTATTATTGGCGACTTTT	14031–14033 of X59554
15	TTGGAACATCGACTTATTGAA	14915–14895 of X59554
16	AGATGTAAAAGGCTGCTTGAT	16373–16393 of X59554
17	ACGTTTGAGCTTCCAATTCTT	16767–16747 of X59554
74	ATAGCGATGTGCTGTGAATT	18136–18155 of X59554
75	CACGGAACTTGATGTATGCT	19298–19279 of X59554
76	TCGACGATTTTTACTGGTTC	19515–19534 of X59554
77	CAGGAATTACAAGCGATCAA	20101–20082 of X59554

[a] The sequences of primers are based on the IS*1004* sequence on pEB1111 or on the sequence of the *V. cholerae* O1 *rfb* cluster (GenBank accession number X59554) (38).

Plasmids were purified with Qiagen columns and sequenced with M13 forward and reversed primers or by dye terminator sequencing (Applied Biosystems) with primers 9, 10, 72, and 73 (Table 3), which correspond to sequences on the repetitive element. All sequence reactions were run and analyzed on an automated DNA sequencer (Applied Biosystems).

Heteroduplex formation. Repetitive sequences in *V. cholerae* were enriched by heteroduplex formation (29, 39). Chromosomal DNA (100 μg) of *V. cholerae* CVD101 was sheared by 20 passages through a 26-gauge needle and precipitated with ethanol. The DNA was resuspended in 50 μl of 50% formamide, denaturated by boiling for 10 min, and allowed to renaturate for 4 h at room temperature. Subsequently, S1 nuclease treatment was performed to remove single-stranded DNA. After ethanol precipitation, the heteroduplex DNA was dissolved in 50 μl of water. Ten microliters was electrophoresed on an agarose gel, Southern blotted, and probed with the insert of pEB1062.

Alignment programs. Sequences were compared by using Multalin or FASTA (30). Alignments of sequences in the EMBL and GenBank databases were performed by using the BLAST program (2).

Standard procedure for *V. cholerae* DNA fingerprinting with IS*1004*. The DNA fingerprinting procedure was performed essentially as described previously (40, 41). Chromosomal DNAs of *V. cholerae* strains were digested with *Hpa*II. After the inactivation of *Hpa*II by placing the digestion mixtures for 20 min at 65°C in DNA sample buffer, a small aliquot was run on an agarose gel to estimate the concentration. Two milligrams of digested DNA of each strain was mixed with 2.5 μl of internal marker. This internal marker contained 1 ng of *Pvu*II-digested supercoiled ladder DNA (BRL) and 0.5 ng of *Hae*III-digested ϕX174 DNA (Boehringer Mannheim) per μl. DNA fragments were separated by overnight electrophoresis on 0.8% agarose gels. DNA was transferred to Hybond-N^+ membranes (Amersham International PLC) by vacuum blotting, and 2-μl aliquots containing both IS*1004* DNA and internal marker in 0.4 M NaOH were spotted on three corners of the filter. Membranes were hybridized with an enhanced chemiluminescence-labeled 624-bp internal fragment of IS*1004*, obtained by PCR of pEB1110 DNA with primers 9 and 10 (Table 3). A second hybridization was performed with labeled internal marker. Because of the presence of spots on the membrane hybridizing to both probes, films could be superimposed very precisely.

Computer analysis of DNA fingerprints. DNA banding patterns on films were imaged by using a Bio-Image Analyzer (Millipore Corporation, Ann Arbor, Mich.). Fingerprints were analyzed with Gelcompar software (Applied Maths, Kortrijk, Belgium). By superimposing the autoradiograms obtained after hybridization with IS*1004* and with internal markers of known molecular sizes, the positions of hybridizing fragments were normalized. Comparison of fingerprints was performed by the unweighted pair group method using arithmetic averages (UPGMA) clustering method by using the Dice coefficient according to the instructions by the manufacturer of Gelcompar.

Nucleotide sequence accession number. The nucleotide sequence of the IS*1004* copy on pEB1003 appears in the EMBL and GenBank databases under accession number Z67733.

RESULTS

Identification of a repetitive sequence in an O1 classical *V. cholerae* strain, CVD101. While characterizing *V. cholerae* transposon mutants affected in the synthesis of δ-aminolevulinic acid (32), we noticed that a *V. cholerae* DNA fragment contained in pEB1062 hybridized to multiple (six to seven) bands on Southern blots, indicating the presence of a repetitive DNA element (Fig. 1). To determine the size of this element, we conducted a heteroduplex experiment (29, 39). Chromosomal DNA of *V. cholerae* was denaturated, renaturated, and treated with S1 nuclease to remove single-stranded DNA. When a Southern blot of the obtained heteroduplex DNA was probed with the insert of pEB1062, a 600-bp band hybridized, defining the approximate size of the repetitive element (Fig. 1).

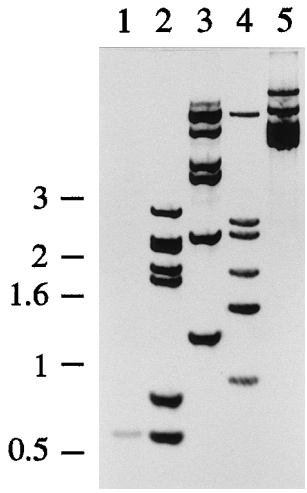

FIG. 1. Southern blot analysis of heteroduplex DNA and restricted chromosomal DNA of *V. cholerae* CVD101. The 300-bp insert of pEB1062 was used as a probe. The positions and sizes (in kilobases) of marker DNA are indicated on the left. Lane 1, heteroduplex DNA; lane 2, *Cla*I digest; lane 3, *Hin*dIII digest; lane 4, *Hpa*II digest; lane 5, *Pvu*I digest.

The DNA sequences of five different copies of the repetitive element were determined, and comparison of these sequences allowed us to delineate the element. The element appeared to comprise 628 bp (Fig. 2A), which corresponded well with the length estimated from the heteroduplex experiment (see above). No terminal inverted repeats nor evidence for target sequence duplication was observed (Fig. 2A). The G+C content of the element was 41%, which is 6 to 8% lower than that of the *V. cholerae* genome (19).

The largest open reading frame contained in the repetitive element, designated *tnpA*, encodes a protein of 145 amino acids, with a predicted pI of 9.99. Database searches revealed that the *tnpA* product showed sequence identities with transposases encoded by the IS element IS*200*, which is found in several bacterial species (7, 22). The highest degree of identity (41%) was found with the transposase encoded by IS*200* from *Salmonella enterica* (Fig. 2B). In addition, we found sequence similarity between the IS*1004*-encoded *tnpA* and an open reading frame located at the end of the hyaluronidase gene of *Streptococcus pneumoniae*, suggesting that this sequence is probably also an IS*200*-like element (5). IS*200* comprises 708 bp and is the smallest IS element known until now (16). Unlike most IS elements but like the *V. cholerae* repeat, IS*200* does not contain terminal inverted repeats or give rise to target DNA duplication (16, 23). On the basis of its similarity to IS*200*, we conclude that the *V. cholerae* repetitive element described here is an IS element, which we have designated IS*1004*.

An incomplete copy of IS*1004* is located in the *rfb* locus of *V. cholerae* serotype O1. A nucleotide sequence comparison of

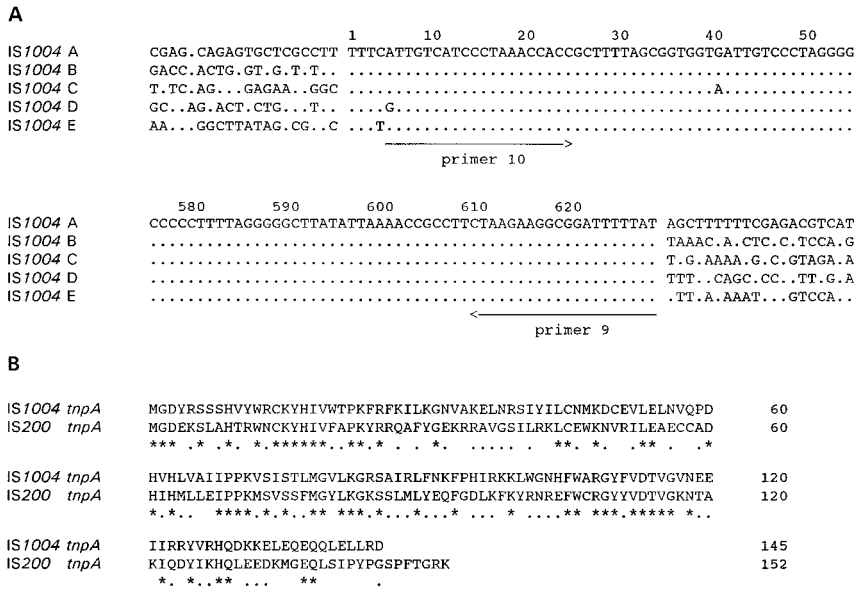

FIG. 2. (A) Alignment of sequences of five cloned copies of IS*1004* (copies A through E) and their flanking regions. Sequence identity with IS*1004* copy A is indicated by a dot. IS*1004* sequences are separated from flanking regions by spaces. Arrows indicate the positions of the primers, 9 and 10, used for generating a probe for DNA fingerprinting. (B) Homology between the putative products encoded by IS*1004* and IS*200* of *S. enterica* (EMBL and GenBank accession number L25848). Asterisks and dots represent identical and similar amino acids, respectively. The percent identity between the two sequences is 40.6%.

IS*1004* with known *V. cholerae* sequences in the EMBL and GenBank databases revealed the presence of an incomplete IS*1004* copy (comprising bases 1 to 190) in the *rfb* locus of *V. cholerae* O1 (24, 38). This region is responsible for the synthesis of the O1 antigen. The IS*1004* sequence is located just upstream of *rfbQRS*, which is also a putative IS element (24). Originally, this region was designated *rfbP* and no homology between the *rfbP* product and any known amino acid sequence was observed (24, 38). However, *tnpA* is located in another reading frame than is *rfbP*, and only the first 22 codons of *tnpA* are present in the incomplete IS*1004* copy. The incomplete IS*1004* copy is visible as a faint band of 3.5 kb in Southern blots of *Hpa*II-digested chromosomal DNAs of most *V. cholerae* O1 strains probed with IS*1004*. This band is not visible in Fig. 1 because the insert of pEB1062, which was used as a probe, does not contain the part of IS*1004* located in the *rfb* region.

IS*1004*-generated fingerprints of epidemic *V. cholerae* serotype O1 and O139 strains. To determine whether IS*1004* could be used to differentiate between epidemic *V. cholerae* O1 strains, we used it as a probe of *Hpa*II-digested chromosomal DNAs of *V. cholerae* O1 strains of classical and El Tor biotypes isolated in different parts of the world (Table 1). We used the restriction enzyme *Hpa*II because this yielded the most even distribution of IS*1004*-hybridizing fragments of the 14 restriction enzymes tested (results not shown). *Hpa*II does not cleave within IS*1004. V. cholerae* O1 strains showed very similar IS*1004* patterns (Fig. 3). All O1 strains tested had IS*1004*-containing *Hpa*II fragments of 1,500, 2,200 and 2,500 bp. A faint band, which in most strains had a size of 3,500 bp, represented the incomplete copy of IS*1004* in the *rfb* locus (see above). The fingerprint patterns of classical biotypes differed from those of El Tor strains in the presence of extra IS*1004* copies and in the decreased mobility of the largest *Hpa*II band. Within the classical and El Tor biotypes, two (C1 and C2) and four (E1 through E4) fingerprint types, respectively, were found. Most (5 of 6) of the classical strains revealed IS*1004* fingerprint type C1, while E1 was the dominant fingerprint type found in the El Tor strains (14 of 29 strains) (Fig. 3; Table 1). All El Tor strains from the Latin American epidemic revealed the same fingerprint, E3. *V. cholerae* O139 strains from the recent epidemic in Asia had pattern E5, which is almost identical to E1, the pattern observed for most El Tor strains, as shown before (6). Only the faint band at 3.5 kb, which represents the incomplete IS*1004* copy in the O1 *rfb* locus, was lacking (Fig. 3, lane 13). This is in agreement with the obser-

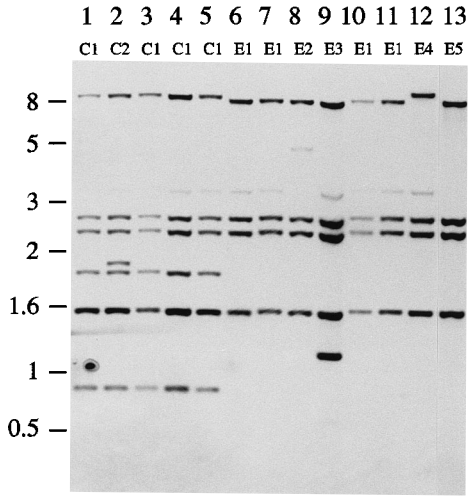

FIG. 3. IS*1004* fingerprints of *Hpa*II-digested chromosomal DNAs of epidemic serotype O1 and O139 *V. cholerae* strains. Lanes 1 to 5, *V. cholerae* O1 classical biotype strains; lanes 6 to 12, *V. cholerae* O1 El Tor biotype strains; lane 13, *V. cholerae* O139 Bengal. The fingerprint type of each strain is indicated below the lane number. Lane 1, 395; lane 2, 569B; lane 3, NCTC 8039; lane 4, C21; lane 5, Cairo 48; lane 6, C5; lane 7, C30; lane 8, N16961; lane 9, 35481; lane 10, 9400378; lane 11, V83; lane 12, CO447; lane 13, MO45. The positions and sizes (in kilobases) of DNA markers are indicated on the left.

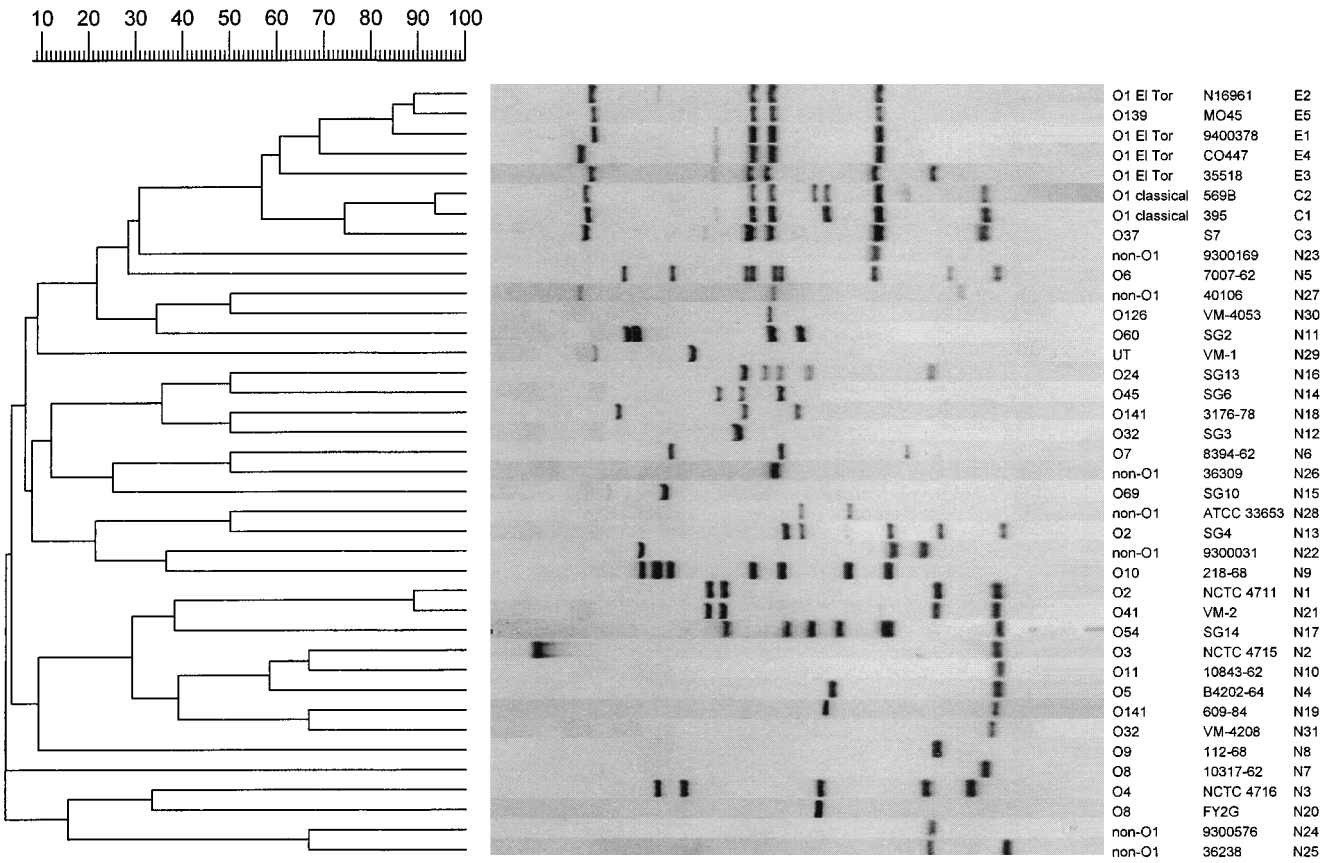

FIG. 4. Clustering of IS*1004* fingerprints of *V. cholerae* strains obtained in this study. Only one strain of each fingerprint type is shown. Strains which did not show hybridization with the IS*1004* probe were not included in this analysis. The codes on the right give the serotype, strain designation, and fingerprint type of each strain. The scale measures similarity values.

vation that O139 strains contain a large deletion in the O1 *rfb* region (6, 24).

IS*1004* fingerprint analysis of O1 and non-O1 *V. cholerae* and *V. mimicus* strains. We extended our fingerprint analyses to include non-O1 and non-O139 *V. cholerae* strains. These strains were environmental and patient isolates, some of which produced cholera toxin (Table 1). Some *V. mimicus* strains were also included, as this species is very closely related to *V. cholerae* and indistinguishable from *V. cholerae* by serology. To be able to compare the banding patterns of different gels, fingerprints were imaged, normalized, and stored in a database. Computer analysis allowed us to cluster related fingerprints. All *V. cholerae* O1 classical and O1 El Tor strains exhibited very similar IS*1004* banding patterns, indicating that they represent a genetically distinct, closely related group (Fig. 4; Table 1). In contrast, the fingerprints of non-O1 and non-O139 strains were very polymorphic and, with one notable exception (see below), unrelated to the banding patterns of classical and El Tor strains (Fig. 4; Table 1). The number of IS*1004*-hybridizing bands ranged from zero to nine. Among 43 non-O1 and non-O139 *V. cholerae* and *V. mimicus* strains, 31 fingerprint patterns (types N1 through N31) were distinguished (Table 1).

Cluster analysis revealed groups of strains with identical or very similar fingerprints that express different O antigens (Fig. 5A). One such group consisted of *V. mimicus* O41 VM-2, *V. cholerae* O2 NCTC 4711, and strain 9300028, a *V. cholerae* non-O1 strain of undefined serotype. O41 and non-O1 strains revealed identical fingerprint patterns (N21), while the pattern of the O2 strain (N1) differed by only one band from this pattern. Another group comprised O139 and O1 El Tor strains, as described above. The most remarkable observation was that the fingerprints of two O37 strains, S7 and G12R, from Sudan were very similar to C1, the predominant pattern in O1 classical strains, differing by only two bands. Therefore, the pattern of these O37 strains was named C3. These results indicate that the O37 and O1 classical strains have a very similar genetic background but harbor distinct cell wall polysaccharide genes. It should be noted that the O37 strains from Sudan produced cholera toxin and were isolated from an outbreak of diarrheal disease in Sudan (43, 44).

In addition to strains which were genetically closely related but expressed different serotypes, we also observed the reverse, i.e., strains from different genetic backgrounds but of identical serotype (Fig. 5B). For instance, the O37 serotype was expressed by strains with (S7 and G12R [see above]) and without (SG8) IS*1004*. Strain SG8 does not contain the cholera toxin genes, in contrast to S7 and G12R (Table 1). Other examples of genetically distinct strains expressing the same serotype include serotype O2 strains NCTC 4711 and SG4, which displayed fingerprint patterns N1 and N13, respectively. Further, seven O141 strains isolated from different regions in the United States showed two different fingerprint patterns. Remarkably, the five O141 patient isolates had the same fingerprint pattern (N19), while the two environmental isolates displayed another pattern (N18). The observation that genetically

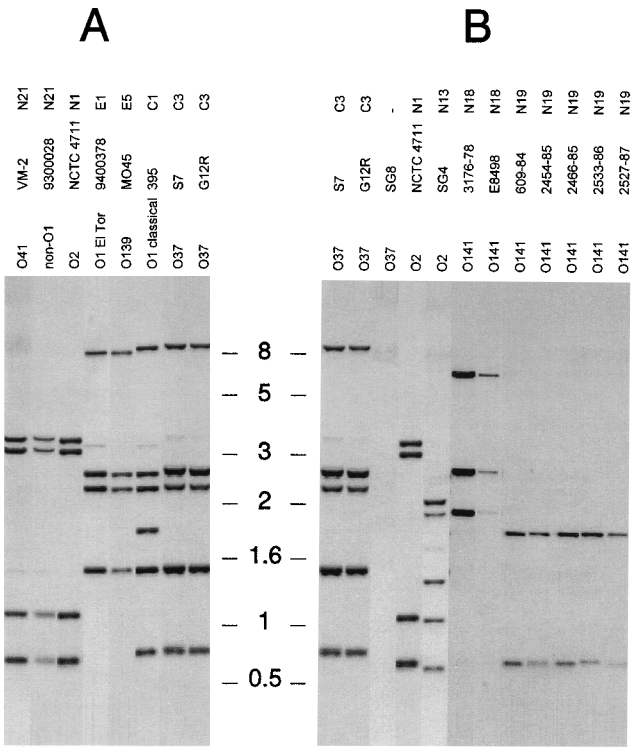

FIG. 5. IS*1004* fingerprints of strains expressing different O antigens but displaying identical or related fingerprints (A) and strains of the same serotype with different fingerprints (B). The positions of molecular weight (in kilobases) markers are indicated in the middle. The codes above each lane are explained in the legend to Fig. 4.

related strains may express different serotypes is consistent with the transfer of cell wall polysaccharide genes in *V. cholerae.*

Characterization of serotype O37 strains S7 and G12R from Sudan. As described above, O37 strains S7 and G12R appeared to be closely related to O1 classical strains. To characterize these strains further, we used fragments obtained by PCR amplification of the *rfb* locus of *V. cholerae* O1 as a probe on Southern blots with O37 chromosomal DNA (Fig. 6). Three fragments containing the central parts of the *rfb* locus, fragments B, C, and D, did not hybridize to chromosomal DNAs of these O37 strains. Probes A and F, however, comprising *rfaD* and *orf2-orf3*, respectively, showed hybridization to these O37 strains, while probe E showed weak hybridization. These results indicate that the two O37 strains from Sudan lack the central part of the O1 *rfb* region, while the flanking regions are present.

Occurrence of IS*1004* among *Vibrio* species other than *V. cholerae.* When the presence of IS*1004* in the genomes of *Vibrio* species other than *V. cholerae* was investigated by Southern blot hybridization, only *V. mimicus* strains hybridized (Table 1). *V. mimicus* is closely related to *V. cholerae*. The number of IS*1004* copies in the six *V. mimicus* strains tested varied from zero to five, and the patterns were as polymorphic as those found in the *V. cholerae* non-O1 strains. *V. fluvialis*, *V. hollisae*, and *V. parahaemolyticus* failed to hybridize (data not shown).

DISCUSSION

In this study, we characterized a new IS element of *V. cholerae*, designated IS*1004*, and we investigated its potential to differentiate *V. cholerae* strains. IS*1004* is 628 bp in size, which makes it the smallest IS element known, and is closely related to IS*200*. IS*200* was first discovered in *S. enterica* (22), but homologous elements have been identified in *E. coli*, *Shigella* spp., *Clostridium perfringens*, and *Yersinia pestis* (7, 8, 15). Like IS*1004*, IS*200* has no terminal inverted repeats and it does not give rise to target sequence duplication (16, 23), two properties frequently associated with other IS elements. IS*200* has proved to be a useful tool for DNA fingerprinting of *Salmonella* serovars (35–37). This study shows that IS*1004* can be applied for studying the genetic relationships of *V. cholerae* isolates.

The number of IS*1004* copies in the *V. cholerae* strains studied varied from zero to eight (Fig. 4; Table 1). In contrast to O1 strains, non-O1 strains displayed very polymorphic patterns, indicating that the latter form a more heterogeneous group. This has also been observed by other genotyping methods (13, 20, 25). Further, the fingerprint patterns of the O1 and non-O1 strains were unrelated, with a few notable exceptions (see below). Our studies revealed that strains displaying the same serotype are not necessarily genetically related. Among the relatively small number (49) of non-O1 *V. cholerae* and other *Vibrio* strains investigated, we found three groups of strains of the same serotype that showed different fingerprint patterns (Fig. 5B). Conversely, we also encountered identical or closely related fingerprint patterns for strains expressing different serotypes (Fig. 5A). Although the first observation could be explained by IS*1004* transposition or sequence divergence resulting in the shift of DNA fragments harboring IS*1004* copies, it is less likely an explanation for different serotype isolates displaying identical or closely related IS*1004* patterns. Rather, our findings indicate that closely related strains may contain gene clusters coding for different serotypes and that horizontal

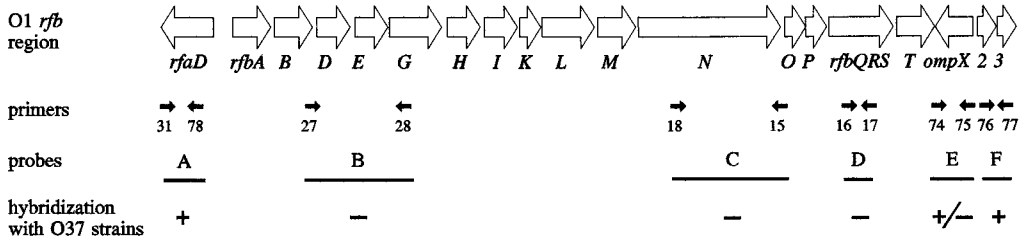

FIG. 6. Presence of serotype O1 *rfb* sequences in serotype O37 strains from Sudan. The O1 *rfb* sequence was derived from those of Stroeher et al. (38) and Manning et al. (24) (GenBank accession number X59554). Open arrows indicate genes; small black arrows indicate primers. Fragments were amplified by PCR on chromosomal DNA of strain C5 with the indicated primer pairs and used as probes on Southern blots of chromosomal DNAs of O37 strains S7 and G12R. +, ±, and −, strong, weak, and no hybridization, respectively.

transfer of O-antigen-determining genes occurs among *V. cholerae* strains.

The high level of polymorphism in *V. cholerae* O antigens suggests diversifying selection. It is not clear what the adaptive value of this diversity is to *V. cholerae* in its natural environment, estuarine systems (11). However, when *V. cholerae* strains interact with the immune system, polymorphism in surface antigens is clearly adaptive. This is dramatically illustrated by the emergence of the O139 Bengal strain. This strain is not affected by immunity to O1 strains, and it is probably not a coincidence that it emerged in a region where naturally acquired immunity to O1 strains is high (1).

In contrast to the non-O1 fingerprint patterns, the O1 patterns were closely related. To facilitate further discussion, fingerprint patterns identical or closely related to those of the O1 classical and El Tor strains are hereafter referred to as classical and El Tor patterns, respectively. Together, the classical and El Tor patterns are designated epidemic patterns. The similarity of classical and El Tor fingerprint patterns indicates that the two biotypes are closely related. Not withstanding this similarity, both biotypes could clearly be distinguished by fingerprinting (Fig. 3). Within the classical and El Tor biotypes, we observed two (C1 and C2) and four (E1 through E4) fingerprint patterns, respectively. El Tor isolates from the Latin American epidemic of 1991 and 1992 could be recognized by their distinct fingerprint pattern (E3). This is in agreement with other reports which showed that strains from the Latin American epidemic are different from the current pandemic strains (i.e., isolates from Asia and Africa) when analyzed by multilocus enzyme electrophoresis or pulsed-field gel electrophoresis (9, 42). Another distinct fingerprint type (E4) was displayed by two El Tor strains isolated in Calcutta in 1994.

Two non-O1 *V. cholerae* strains with an epidemic fingerprint pattern, the O139 Bengal strain (isolates MO45, NT552, and NT554) and the O37 Sudan strain (isolates S7 and G12R), were observed. The close genetic relationship between the Bengal strain and the O1 El Tor strain has been described previously (6, 12, 24). Serotype O37 isolates S7 and G12R were isolated from a large outbreak of diarrheal disease in 1968 in Sudan (43), one of the largest outbreaks caused by a non-O1 strain other than O139 Bengal. Further, it was shown that strain G12R produced cholera toxin (44) and that pili of O37 strain S7 were immunologically indistinguishable from the 16-kDa pili of O1 strains (28). Viewed in retrospect, these observations can be explained by our finding that the O37 strains from Sudan are genetically closely related to epidemic O1 strains. In 1968, the Sudan strain was classified as a noncholera vibrio because it did not agglutinate with the O1 serum. This exemplifies the pitfalls of using phenotypic methods to differentiate *V. cholerae* strains. IS*1004* DNA fingerprinting revealed that the Sudan strains are actually closely related to the O1 classical lineage. Thus, both El Tor and classical fingerprint type strains may carry non-O1 antigens. We propose to change the designations of the G12R and S7 strains to *V. cholerae* O37 Sudan.

Like the O139 Bengal strain, the O37 Sudan strains lack most of the O1 *rfb* cluster (Fig. 6), but they do contain genes flanking this region. It is conceivable that after horizontal transfer of cell wall polysaccharide genes between *V. cholerae* strains, such conserved flanking regions are used for recombinational exchange. Such a mechanism would target the genes to a particular position in the chromosome and avoid the presence in a strain of two gene clusters encoding distinct cell wall polysaccharides. Interestingly, the O37 serotype is also expressed by a strain unrelated to the epidemic lineages, i.e., SG8. The relationship between the O37 Sudan strain and O1 classical strains is unclear. The two lineages may have evolved from each other directly by exchange of cell wall polysaccharide genes, in which case either one could be the progenitor. It is also possible that the two lineages have diverged from a common ancestor with a distinct (non-O1 and non-O37) serotype. Seven cholera pandemics have been distinguished; only strains from the last three have been isolated, and they were identified as O1 strains. Possibly, older epidemics were caused by strains with an epidemic genotype expressing non-O1 serotypes.

The identification of a second non-O1 strain with an epidemic genotype clearly demonstrates the usefulness of DNA fingerprinting in studying the epidemiology of cholera. It is conceivable that widespread use of this method and other genotypic methods will identify more such strains and increase our knowledge of the evolution of epidemic *V. cholerae* strains.

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